

# Enhancement of the immunogenicity of a synthetic peptide bearing a VP3 epitope of hepatitis A virus

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**Abstract** The immune responses elicited in mice by different forms of the VP3(110–121) B-epitope of the hepatitis A virus (HAV) were studied. Different forms of incorporation in liposomes were tested, encapsulation, rather than surface exposure, being the best antigenic preparation. Three larger peptides of the VP3 epitope, two of them containing a hepatitis B virus T-epitope, and a third containing a putative T-epitope of HAV (VP3(102–121)) were assayed. While this latter T-epitope induced an enhancement of the response against the VP3 B-epitope, the artificially coupled T-epitopes failed to induce a significant increase. The administration of two multiple antigenic peptide (MAP) constructs, the first containing the VP3(110–121) and VP1(11–25) HAV sequences and the second only the VP1(11–25) sequence, also suggested the presence of a T-epitope, since the response against the VP1 peptide was increased in the first construct.

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**Key words:** Hepatitis A virus; B-epitope; T-epitope; Synthetic peptide; Liposome

## 1. Introduction

Hepatitis A virus (HAV) is a hepatotropic virus which is classified as genus *Hepatitisvirus* within the Picornaviridae family [1]. The virion capsid is composed of structural proteins: VP1, VP2, VP3, and, possibly, VP4 [2]. Although hepatitis A is not considered a severe disease, it continues to be a source of mortality in both developed and developing countries [3].

The developed formalin-inactivated HAV vaccines [4,5] represent a substantial step forward in the control of infections in developed countries. However, the poor yields of HAV in cell cultures [6] cause high production costs and pose serious difficulties to the use of this vaccine in developing areas. As an alternative, the use of new strategies based on synthetic peptides which can elicit an efficient immune response offers the advantage of high purity, defined structure and safety [7,8]. However, the use of synthetic peptides as HAV immunogens presents an important difficulty since the major viral neutralisation epitopes appear to be discontinuous [9]. Despite this fact, one sequence exists in the VP1 protein [10,11] and one

sequence in the VP3 protein [12] susceptible to be used as HAV immunogens. The immunogenicity of the VP1(11–25) sequence has been described elsewhere [11], and in the present work we will present the immunogenicity of the VP3(110–121) sequence. One of the major unsolved problems of the use of synthetic vaccines is that they usually are poorly immunogenic and often require vehicles or carriers to evoke an immune response. In this study, different delivery systems are tested in order to enhance the immunogenicity of the VP3 peptide such as liposomes and synthetic amino acid polymers.

## 2. Materials and methods

### 2.1. Cells and viruses

FRhK-4 cell cultures were used to propagate and assay the cytopathogenic HM-175 (courtesy of T. Cromeans, Centres for Disease Control, Atlanta, GA, USA) strain of HAV [13]. Viral enumerations were performed by calculating the most probable number of cytopathogenic units per ml (MPNCU/ml) by infecting cell monolayers grown in 96-well microtitre plates [14]. Sixteen wells were infected for each dilution, and 20 µl of inoculum was added to each well. Data were processed with an MPN computer programme [15].

### 2.2. Synthetic peptides

Peptides containing the VP3(110–121) (FWRGDLVFDFQV) epitope were synthesised by the continuous-flow Fmoc-polyamide solid-phase method [16]. Peptides were purified by semi-preparative HPLC and the purity achieved was greater than 95%. To enhance the immunogenicity of the VP3 epitope, this sequence was administered in several forms: combined with liposomes, combined with T-epitopes and liposomes, and as multiple antigenic peptide (MAP) constructs. Three liposome incorporation approaches were employed: entrapment into liposomes (liposomes-VP3), entrapment into liposomes after derivatisation with palmitic acid (liposomes-palmitoyl-VP3), and covalent binding to liposomes (liposomes-covalent-VP3). Three T-epitope constructs were also assayed: a longer peptide incorporating at the amino termini a 15-mer peptide (HBsAg) derived from an anti-idiotypic sequence of the hepatitis B virus surface antigen that contains B- and T-epitopes (Anti-id 2F10) [17] and entrapped into liposomes (Anti-id 2F10-VP3), a second construct incorporating the Anti-id 2F10 at the carboxy termini and entrapped into liposomes (VP3-Anti-id 2F10), and finally a longer VP3 peptide entrapped into liposomes VP3(102–121). This larger sequence of the natural HAV VP3 protein is predicted to contain a T-epitope following the amphipathic helical profile of the protein and in foot-and-mouth disease virus (FMDV) contains a T-epitope [18]. MAP constructs employed were MAP<sub>4</sub>-VP3, incorporating 4 molecules of VP3(110–121) and MAP<sub>2-2</sub>-VP3-VP1, incorporating 2 molecules of each VP3(110–121) and VP1(11–25). A MAP<sub>4</sub>-VP1 was also administered to perform comparative studies with the VP3/VP1 construct. All these forms are summarised in Figs. 1–3.

### 2.3. Antibody production

Six-week old female Swiss mice were used to obtain ascitic antibodies, after immunisation with the different synthetic peptides employing Freund's complete adjuvant (FCA) as enhancer [11]. The peptide preparations were administered diluted 1:10 in FCA, in five

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**Abbreviations:** HAV, hepatitis A virus; MPNCU/ml, most probable number of cytopathogenic units per ml; HBsAg, hepatitis B virus surface antigen; FCA, Freund's complete adjuvant; ELISA, enzyme linked immunosorbent assay; MAP, multiple antigenic peptide; FMDV, foot-and-mouth disease virus; BSA, bovine serum albumin

doses of 50 µg of peptide each, at weeks 0, 2, 3, 4 and 5. Ascites generated by inoculation of PBS diluted 1:10 in FCA were used as negative controls, while ascites generated by inoculation of 30 ng of intact HAV particles were used as positive controls.

#### 2.4. Peptide recognition

Peptide recognition by anti-peptide antibodies was assayed by a direct enzyme-linked immunosorbent assay (ELISA), in which plates were coated with the VP3(110–121) peptide or the VP1(11–25) peptide (1 µg/well) and then reacted with ascitic fluids generated against each of the peptide formulations. The antigen-antibody complex was detected using an enzyme-labelled goat anti-mouse serum. Minimal positive ELISA (cut-off) values were calculated by adding 2 standard deviations to the mean of tested reference negative ascites.

A competitive test was employed to assay the recognition of the synthetic peptides by a human convalescent serum. The serum was preincubated for 2 h at 37°C with either 10 µg of free peptide or bovine serum albumin (BSA) in an equimolecular ratio per ml of serum, and tested for HAV recognition in a sandwich ELISA. HAV or FRhK-4 cell lysates were captured through MAb 33Z/37/39 and detected by means of the convalescent serum. An enzyme-labelled anti-human serum was employed to detect the antigen-antibody complex. A statistically significant ( $p < 0.05$ ; ANOVA test) decrease in HAV recognition by the convalescent sera preincubated with HAV-related peptides with regard to the same sera preincubated with BSA was indicative of peptide recognition.

#### 2.5. Virus recognition

HAV recognition by anti-peptide antibodies was tested by a competitive ELISA, consisting of a preincubation of ascitic fluids with intact HAV virus ( $5 \times 10^5$  MPNCU per ml of sample) for 2 h at 37°C, before being added onto the immobilised peptides. Supernatants from mock-infected FRhK-4 lysates were incubated with ascites and used as negative controls. A statistically significant ( $p < 0.05$ ; ANOVA test) decrease in peptide recognition by the anti-peptide ascites preincubated with HAV with regard to the same ascites preincubated with mock-infected cell lysates was indicative of virus recognition.

HAV recognition by antibodies generated using the virions as antigen was evaluated by a sandwich ELISA. Viruses or mock-infected cell lysates were captured by a convalescent serum, and detected by the ascitic fluids. Minimal positive ELISA (cut-off) values were calculated by adding 2 standard deviations to the mean of optical densities obtained with the mock-infected cell lysates.

#### 2.6. Virus neutralisation

Ascitic fluids were assayed for their capacity to neutralise the infectivity of HAV suspensions ( $3.5 \times 10^5$  MPNCU/ml) after a 3-h incubation at 37°C, as described elsewhere [11].

All experimental procedures were performed at least in triplicate.

### 3. Results and discussion

The HAV-VP3(110–121) sequence has recently been described to contain a new continuous B-epitope of the virus [12]. The efficacy of liposomes as vehicles has been clearly established for a great variety of antigens such as bacterial toxoids [19,20], parasite proteins [21] and tumour antigens [22]. It has been speculated that the attachment of peptides to the surface of liposomes, instead of simple encapsulation, could increase its immunogenicity [23]. In the present study, neither the incorporation of the peptide as a lipopeptide, and

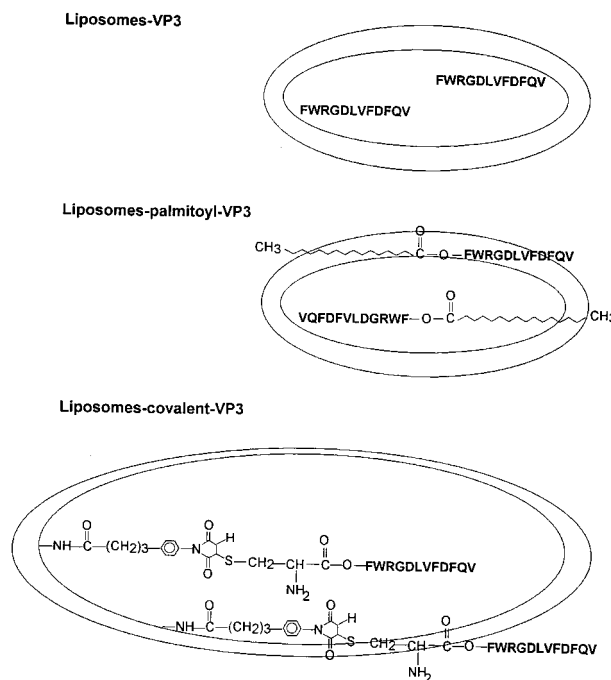


Fig. 1. Different forms of incorporation in liposomes of the VP3(110–121) peptide.

thus potentiating its interdigitation between the lipid bilayers, nor its direct incorporation into the bilayer through a covalent bond with phospholipids, enhanced its immunogenicity (Table 1). On the contrary, the anti-peptide response was of lower magnitude, particularly with the palmitoyl-VP3 form, indicating a potential change on the B-epitope configuration since the level of peptide incorporation into liposomes was higher than the level obtained by entrapment of the unmodified peptide. Since the superficial localisation of the liposome did not increase the potential immunogenicity of the VP3 epitope, entrapment into liposomes was the method of choice to deliver VP3-modified sequences.

Combination of T- and B-cell epitopes in a synthetic peptide, either by selecting sequences that naturally contain such epitopes in proximity, or by artificially coupling B- and T-cell epitopes that lie apart in the natural sequence or even belong to different proteins, has been suggested to be a useful approach to enhance the immunogenic properties of synthetic peptides [24,25]. Two hundred and fifty µg of the longer VP3 peptides were administered entrapped into liposomes, which represents 100 µg of the B-epitope for the Anti-id 2F10-VP3 and VP3-Anti-id 2F10 peptides and 150 µg for the VP3(102–121) peptide. The VP3-Anti-id 2F10 peptide exhibited a little enhancement of the humoral response against the HAV-B-epitope with regard to the VP3(110–121) peptide, since a lower dose of the B-cell epitope was given, and exerted its effect by increasing the anti-HAV titre, although in a much lower proportion of animals (Table 2). On the contrary, the Anti-id 2F10-VP3 peptide did not show any enhancement effect since lower anti-peptide titres were achieved than with the VP3 sequence alone, and even no anti-HAV response was obtained (Table 2). It has been described that chimeric peptides composed of a T-cell epitope linked to the amino terminus of a B-cell epitope induce the production of higher-affinity antibodies than does immunisation with peptides containing

Table 1  
Immunogenicity of different liposome-VP3(110–121) preparations

Product	Maximum anti-VP3 titre	P/T <sup>a</sup>
Liposome-entrapped VP3	1/10 000	3/6
Liposome-palmitoyl VP3	1/100	1/6
Liposome-covalent VP3	1/100	2/6

<sup>a</sup>Ascites positive for peptide recognition/total ascites.

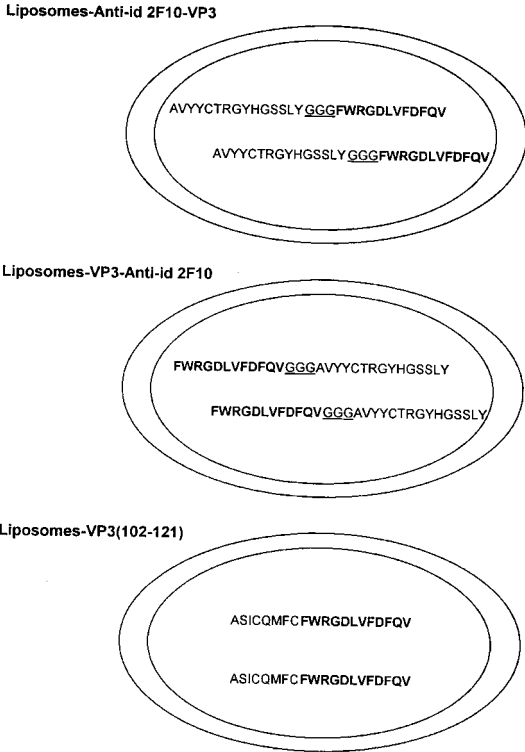


Fig. 2. Peptides containing the VP3(110–121) sequence and a putative T-cell epitope, entrapped into liposomes. Bold amino acid sequences correspond to the B-cell epitope, non-bold sequences correspond to the T-cell epitopes and underlined sequences correspond to linker amino acids.

the epitopes in the opposite orientation [26]. However, in our case a worse response was evoked by the amino terminus location of the HBV-derived T-cell epitope, which may be due to the potentially different conformations adopted by the HAV-B-cell epitope in each case. In similar studies performed with a B-cell epitope of the FMDV, the position of another HBV-derived T-cell epitope did not influence the response [27]. The VP3(102–121) peptide showed an important enhancement of the immunogenicity of the HAV-B-epitope in terms of antibody titre, number of responding animals and duration of this response, being of higher magnitude than the one observed with the VP3-Anti-id 2F10 sequence. The anti-HAV titres achieved with the VP3(102–121) peptide were as high as 1:2000, which means 100 times higher than the response obtained with the VP3(110–121) (Table 2). The proportion of responding animals also increased (83% vs. 50%). The recognition of both peptides VP3(110–121) and VP3(102–121) by a human convalescent serum was similar, which implies that the B-epitope contained in both sequences should be identical. This recognition was evaluated as the percent inhib-

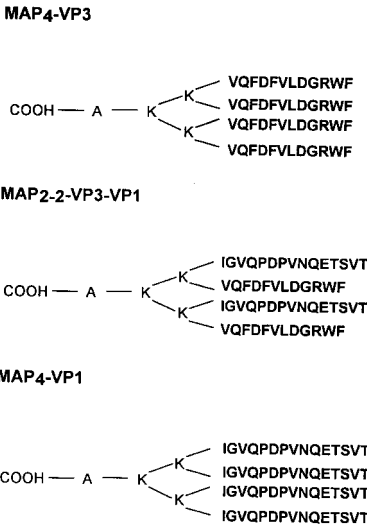


Fig. 3. MAP constructs containing the VP3(110–121) sequence and/or the VP1(11–25) sequence.

ition of human convalescent serum binding to HAV by the different peptides in comparison to an equimolecular amount of an unrelated protein as BSA. The inhibition achieved was around 80% and 70% with the VP3(102–121) and the VP3(110–121) peptides, respectively. Additionally, the VP3(102–121) peptide was recognised by the anti-VP3(110–121) at the same level that these antibodies recognised the VP3(110–121) sequence and, vice versa, antibodies generated with the VP3(102–121) peptide recognised both peptides equally. All these results suggest the presence in this VP3 region of a natural T-epitope adjacent to the B-epitope. This possibility is currently being investigated. Linkage of B- and T-cell epitopes has been described in other viruses [28] and suggests that their close proximity is a factor in determining the efficiency of the antibody response. The putative natural T-cell epitope of HAV is located at the amino terminus of the B-cell epitope.

Synthetic polymers of selected amino acids such as MAP have been used as carriers for different epitopes [29,30]. This system presents the advantage of the possibility of incorporating different epitopes, or several copies of an epitope in the same molecule. In the present work 250 µg of the free MAP polymers were assayed. The responses against both the VP3 and VP1 epitopes were very weak with any of the MAP constructs tested (Table 3). These lower responses in comparison with the responses induced by the same sequences when administered incorporated into liposomes are likely due to the loss of the capacity of induction of cellular immunity by liposomes [31]. However, the anti-VP1 response generated by the MAP<sub>2-2</sub>-VP3-VP1 was significantly higher than the anti-VP1

Table 2  
Immunogenicity enhancement of the VP3(110–121) peptide entrapped into liposomes by administration of larger peptides containing T-epitopes

Product	µg VP3(110–121) sequence	Maximum anti-VP3 titre	P/T <sup>a</sup>	Maximum anti-HAV titre	P/R <sup>b</sup>
Anti-id 2F10-VP3	100	1/1000	2/6	—	0/2
VP3-Anti-id 2F10	100	1/4000	5/6	1/100	2/5
VP3(102–121)	150	1/5000	5/6	1/2000	5/5
VP3(110–121)	250	1/10 000	3/6	1/20	3/3

<sup>a</sup>Ascites positive for peptide recognition/total ascites.  
<sup>b</sup>Ascites positive for HAV recognition/ascites positive for peptide recognition.

Table 3  
Immunogenicity of the VP3(110–121) and VP1(11–25) peptides administered as MAP

Product	Maximum anti-VP3 titre	P/T <sup>a</sup>	Maximum anti-VP1 titre	P/T <sup>b</sup>
MAP <sub>4</sub> -VP3	1/100	1/6	–	–
MAP <sub>4</sub> -VP1	–	–	–	0/6
MAP <sub>2–2</sub> -VP3-VP1	1/100	1/6	1/100	4/6

<sup>a</sup>Ascites positive for VP3(110–121) recognition/total ascites.

<sup>b</sup>Ascites positive for VP1(11–25) recognition/total ascites.

Table 4  
Comparative immunogenicity of HAV particles with the different VP3 sequences entrapped into liposomes

Product	µg total antigen	µg VP3(110–121) sequence	Anti-HAV titre	Neutralisation titre <sup>a</sup>	Neutralisation P/T <sup>b</sup>	Duration of response (weeks)
HAV	0.030	0.00050	1/100 000	1/2000	5/5	> 5
VP3(110–121)	250	250	1/20	1/4	3/6	3
VP3(102–121)	250	150	1/2000	1/25	5/6	> 5

<sup>a</sup>Maximum titre for 90% neutralisation.

<sup>b</sup>Ascites positive for neutralisation/total ascites.

response generated with the inoculation of the MAP<sub>4</sub>-VP1 (Table 3). It should also be noted that a 500-µg dose of the MAP<sub>4</sub>-VP1 polymer [11] was required to induce a similar anti-VP1 response as that of 250 µg of the MAP<sub>2–2</sub>-VP3-VP1 polymer, which contains only 125 µg of the VP1 sequence. These results also suggest the potential presence of a T-epitope, which, however, would be incomplete, in the VP3 sequence, which acts on the VP1 sequence. In fact, this VP3(110–121) sequence also aligns with the FMDV T-cell epitope, with the exception of one residue from the amino terminus of the FMDV sequence which is lost in the HAV peptide [32]. The HAV-VP3(102–121) peptide contains the complete sequence of the putative T-cell epitope. From a structural point of view, the (110–121) sequence would fit with the βD-sheet of the entire VP3 protein while the (102–121) sequence would fit with the αA2-helix-βD-sheet of the entire VP3 protein [32], and if this is also correct for the peptides, the last conformation may contain an amphipathic helical structure that may correlate with the localisation of a T-cell determinant [33].

Since only the VP3(102–121) peptide induced an enhanced response with regard to the VP3(110–121) peptide, comparative studies between these two sequences and intact HAV virions were performed, in terms of antigen dose, percent of responding animals and duration of the response. The immunogenic response induced by the VP3(110–121) and VP3(102–121) sequences administered entrapped into liposomes in comparison with that of the intact HAV virion is depicted in Table 4. Although the immune responses elicited by the VP3(110–121) and VP3(102–121) sequences entrapped into liposomes are of much lower magnitude than those of the intact particles, since only one epitope is used which is not the immunodominant site of the virion, it is relevant to point out that a significance enhancement in the immune response, in terms of titres and duration of response, is achieved simply by increasing the length of the sequence. Future developments include the synthesis of constructs containing the VP3(102–121) and the VP1(11–25) sequences incorporated into liposomes, with the aim of obtaining a synthetic vaccine formulation containing more than one epitope.

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